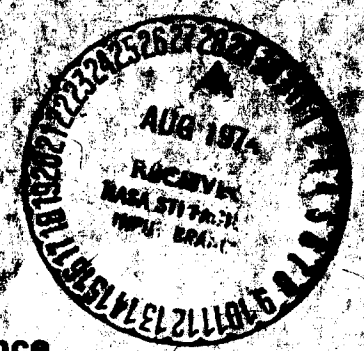
# BIOPHYSICAL RESEARCH LABORATORY

## SIXTEENTH ANNUAL REPORT

JEROME J. WOLKEN

NGR-39-002-011



Mellon Institute of Science
Carnegie-Mellon University
Pittsburgh, Pennsylvania 15213



JUNE 11, 1971

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XVI Annual Report

1970 -- 1971

BIOPHYSICAL RESEARCH LABORATORY

# I. Introduction

The Biophysical Research Laboratory was founded in 1953 at the Eye and Ear Hospital, University of Pittsburgh Medical Center. The Laboratory, since 1964, has been at Carnegie-Mellon University as an integral part of its program in the biological sciences.

The purpose of the Laboratory is to carry on a program of research in Photobiology. The principal area of research for the past seventeen years has been directed toward the study of photoreceptor systems. That is, in search for a better understanding of the nature of photosynthesis, vision and the photobehavior of plants and animals.

Our approach to unravelling how the photoreceptor systems function in living plants and animals has been through the following methods: (1) a quantitative study of the excitatory physical forces, the stimuli; (2) a comparative study of the molecular structure of the photoreceptors; (3) a chemical study of photoreceptor pigment molecules, their biosynthesis and their molecular structure; and (4) a study of the photochemistry of the photoreceptor pigments.

As living model systems to study these phenomena, a variety of organisms are being used. Also as models, mechanical, chemical and electronic devices are being designed and developed that have behavioral properties analogous to that of the photoreceptor systems being investigated.

The results of our studies are recorded in 100 research papers published in scientific journals, 5 books, 2 edited works, and in these Annual Reports of the Biophysical Research Laboratory, volumes I-XVI, 1953 to 1971.

In these Annual Reports, our year-by-year experimental findings are recorded and the bits of information that have been gathered are summarized. In addition, these Reports indicate the personnel responsible for the research, the experimental methods, and the philosophy under which the research is carried out.

## II. Biophysical Research Laboratory Staff, 1970-1971

| | |
|---|---|
| Wolken, Jerome J. | B.S., M.S., Ph.D., University of Pittsburgh; Principal Investigator; Professor of Biophysics. |
| Ootaki, Tamotsu | B.S., Yamagata University; M.S., Ph.D., Nagoya University, Japan; Visiting Research Fellow. From July 1970. |
| Schultz, Alfred J., Jr. | B.S., Pennsylvania State University; Ph.D., University of California, Berkeley; Visiting Research Fellow. From December 1970. |
| Gallik, Gerald J. | B.S., University of Pittsburgh; M.S., Carnegie-Mellon University; Research Biologist. April 1961 to August 1970. |
| Florida, Robert G. | B.S., University of Pittsburgh; Research Biologist. December 1958 to April 1970. |
| Dubash, Pervez J. | B.Sc. and Ph.D., University of Bombay; Visiting Research Fellow from University of Bombay. January 1969 to May 1970. |
| Bashor, Oliver J., Jr. | Technician. |
| Mann, Arlene R. | B.A., M.A.T., University of Pittsburgh; Secretary/Editorial Assistant. |

### A. Graduate Students and Research Assistants

| | |
|---|---|
| Kolodner, Richard | Biology; University of California, Riverside. |
| Petersen, Christopher A. | B.S., Biology; B.A., Psychology; Carnegie-Mellon University. |
| Quick, Frank | B.S., M.S., Electrical Engineering; Carnegie-Mellon University. |
| Wolken, A. Jonathan | B.A., Philosophy; Dartmouth College. |

## III. Research Progress

Although important advances have recently been made into the biochemical nature and structure of living cells, little is known of how cells perceive external stimuli -- the environmental information it needs to behave as a whole integrated organism.

In an attempt to comprehend at the molecular level how visible light initiates photobehavior of plants and animals, and how this is related to our understanding of photosynthesis, vision and nervous excitation, our research has been directed toward discoveries in the physics and chemistry of photoreceptor systems. That is, to find out how the molecular structure of photoreceptors and their photosensitive pigments function in the process.

The photoreceptors which have evolved throughout all plant and animal phyla are differentiated structures, organelles, of the cell. For example, the chloroplast of plant cells for photosynthesis, and the retinal rods and cones of the animal eye for vision -- are organized in a specific molecular geometry. The key aspect of this geometry is that each photoreceptor structure is a highly ordered matrix of repeat units similar to what is found in a crystal. Experimental evidence is now beginning to show that the photoreceptor function is related to its molecular state; for example, as a "liquid crystalline" system which undergoes phase transitions, depending on its constituents (pigment, protein and lipid) and the environmental conditions, particularly light.

The photoreceptor pigment molecules in such a crystalline matrix behave as sensors and possess in a sense "photochemical memory," a phenomenon which allows information (a light signal of a particular wavelength) to be coded into the structure of the molecule. Information then can be introduced by exposure to light of that particular wavelength, "erased" by light of another wavelength, and "recalled" by exciting with the original light signal. For example, the pigment phytochrome, which controls the flowering of plants, depends upon the wavelength of light in the red part of the spectrum to initiate the event.

All animals that possess eyes share a common chromophore molecule for visual excitation which is complexed with a specific protein to form a series of visual pigments called rhodopsins. The precursor for the visual pigment is synthesized by plants, by polymerization of isoprene units into a long

polyene chain of forty carbon atoms, $C_{40}$, which is β-carotene (Figure 1a). In animals, this chain of forty carbon atoms is split in half to two chains of twenty carbon atoms, $C_{20}$, which is recognized as Vitamin A (Figure 1b). In the scheme of evolution, animal cells had to evolve specific enzymes to split the forty carbon atoms into two twenty carbon chains; hence, the probable evolution from plant $C_{40}$ (β-carotene) → $C_{20}$ (Vitamin A). The visual pigment, chromophore, is a carotenoid molecule known as retinal (Figure 2), which interestingly enough is the aldehyde of Vitamin A. The spectra for Vitamin $A_1$, $retinal_1$, and β-carotene are shown in Figure 3.

Another important class of pigments found in plants and animals are the flavines, the most familiar of which is Vitamin $B_2$, riboflavin (Figure 4 and its visible spectrum in Figure 3). Experimental evidence indicates that the photosensitivity of flavine complexes and flavoproteins are also essential to a large number of closely related photoprocesses in living cells.

In search of answers to how photoreceptor systems developed, how they are structured for light capture, and how they function as an integrated system, it seemed that some of the answers would be found among the microorganisms that border between plants and animals, as in the unicellular euglenoids and in the fungi. Therefore, our research efforts during this past year were directed to the photobehavior of the fungus, Phycomyces blakesleeanus. The sporangiophore of Phycomyces is a single cell some centimeters in length. The cell reacts to a variety of external stimuli, and therefore, we have selected it as a model cell system for photobehavior and neurosensory studies.

FIGURE 1.

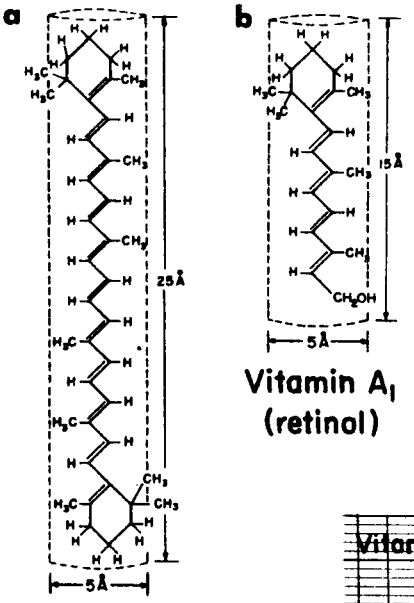
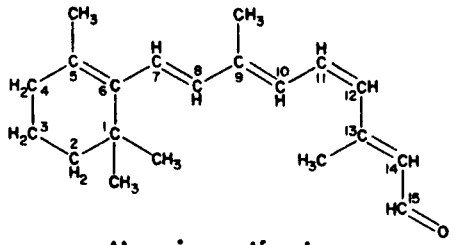



FIGURE 2.



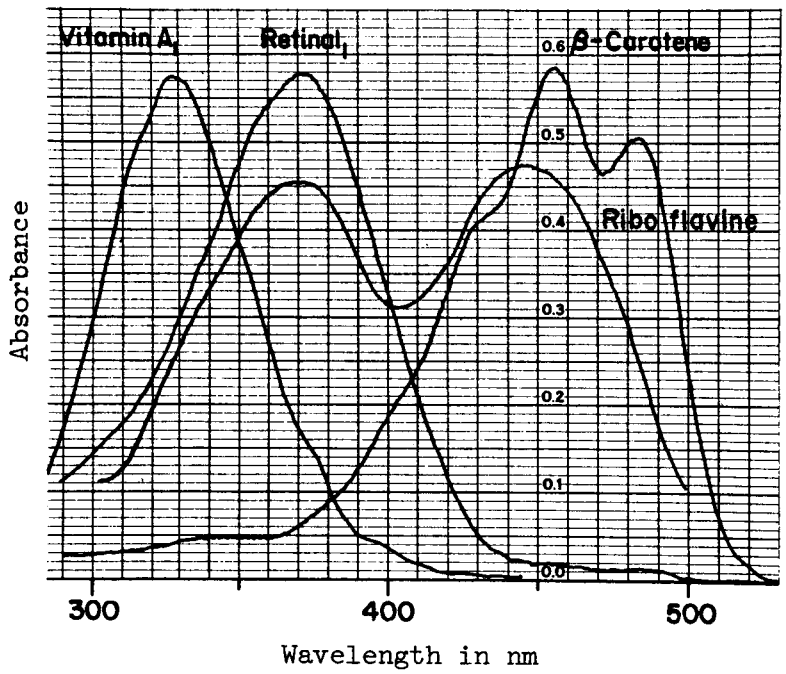




FIGURE 3. Absorption spectra of Vitamin $A_1$, Retinal$_1$, β-carotene and Riboflavine (Vitamin $B_2$).

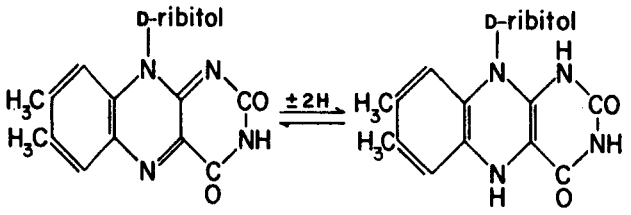
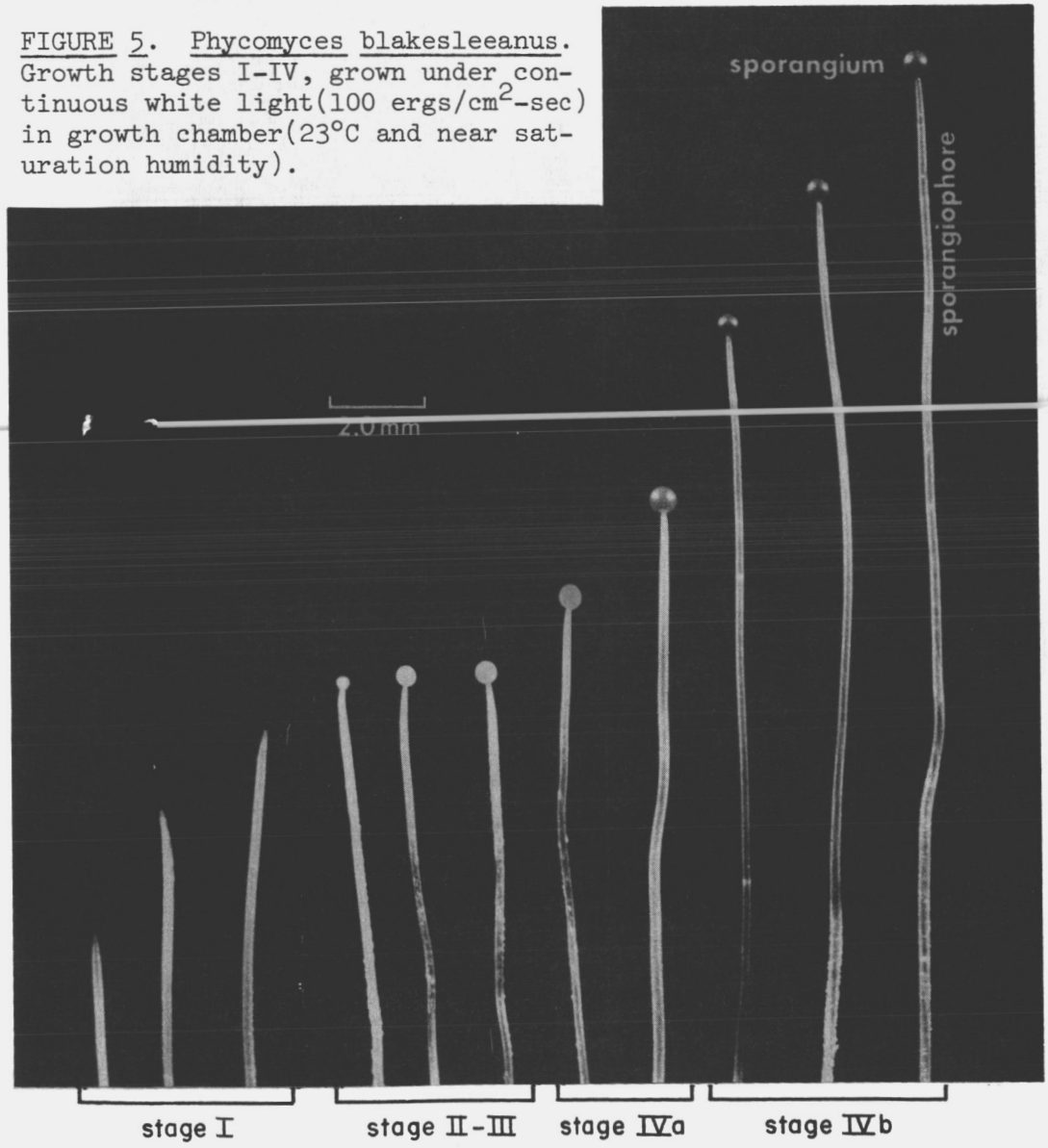



FIGURE 4.

A. Phycomyces: A Model Photosensory Cell. J. J. Wolken.

The growth of Phycomyces follows four distinct phases which are described as stages I through IVb (Figure 5). The sporangiophore which is a large single cell is most sensitive to light (in stages I and IVb) and to other physical stimuli. The sporangiophore exhibits two light-sensitive phenomena, that of phototropism and a light-growth response. Light is not essential for growth but functions as a signal to alter the growth in either space or time.

FIGURE 5. Phycomyces blakesleeanus. Growth stages I-IV, grown under continuous white light(100 ergs/cm$^2$-sec) in growth chamber(23°C and near saturation humidity).



Although Phycomyces has long been the object of research concerned with the sporangiophore's sensitivity to light, no photoreceptor structure has been identified and it is not precisely known what pigment or pigment system is responsible for these light responses.

Action spectra for phototropism and the light-growth response show absorption bands around 280, 365-385, 420-425, 445-485 nm. These action spectra indicate that the photoreceptor molecules for both phenomena should be similar. The absorption peaks in the visible part of the spectrum coincide with that of β-carotene. The absorption peaks in the ultraviolet near 280 and 370 nm and in the visible around 450 nm of the action spectrum suggested that the photoreceptor molecule is a flavine.

To learn more about whether the behavioral action spectrum has meaning, the following studies were undertaken: electron microscopy of the sporangiophore in search of a photoreceptor structure; chemical extraction of the sporangiophore in attempts to isolate a pigment or pigment system; and microspectrophotometry in the light-growth zone to identify from the absorption spectra the pigments responsible.

Phycomyces wild-type and two albino mutant strains, car-5 type I and car-10 type II, which are deficient in carotenes, particularly β-carotene, were used in these studies. The albino car-5 is of special interest, for it is "night blind," in the sense that it is phototropic only at high light intensities.

The fungi were grown on Sabouraud potato dextrose agar (Difco) in a temperature-controlled growth chamber, 22°C, under continuous fluorescent light of either 65 or 30 foot candles and in darkness at a relative humidity near 50%, in petri dishes 100 x 20 mm. The absorption spectra of the sporangiophore was obtained with a recording microspectrophotometer M-5. This instrument is designed to rapidly record absorption spectra in a single sweep at relatively low light levels, from the ultraviolet, 220 nm, through the visible to 800 nm in the infrared. For microspectrophotometry, sporangiophores were plucked from the mycelia, mounted on a quartz microscope slide in air and covered with a quartz cover slip to avoid crushing them. Cytoplasmic streaming continued during the measurements. The general appearance

of the sporangiophore in stage IVb is a nearly transparent cylindrical filament supporting a spherical black sporangium.

The absorption spectra of stage IVb of the wild-type sporangiophore from the sporangium through the growing-zone (within 3 mm) is shown in Figure 6a,b. The spectrum of Figure 6a is typical of what is found from the bottom of the sporangium through 1 mm in the growing-zone. Scanning down the sporangiophore, from 1 mm to 2 mm below the sporangium, the absorption spectrum gradually shifts from the spectrum shown in Figure 6a to that of Figure 6b. The spectrum of Figure 6a is similar to a carotene and its absorption peaks are very near to those of β-carotene at 430, 460 and 480 nm (Figure 1a). It is interesting to note that the absorption peaks of Figure 6a and b together give absorption peaks at 280, 370, 435, 460 and 485 nm, which are found in the complete phototropic action spectrum.

Microspectrophotometry
Phycomyces blakesleeanus, wild-type

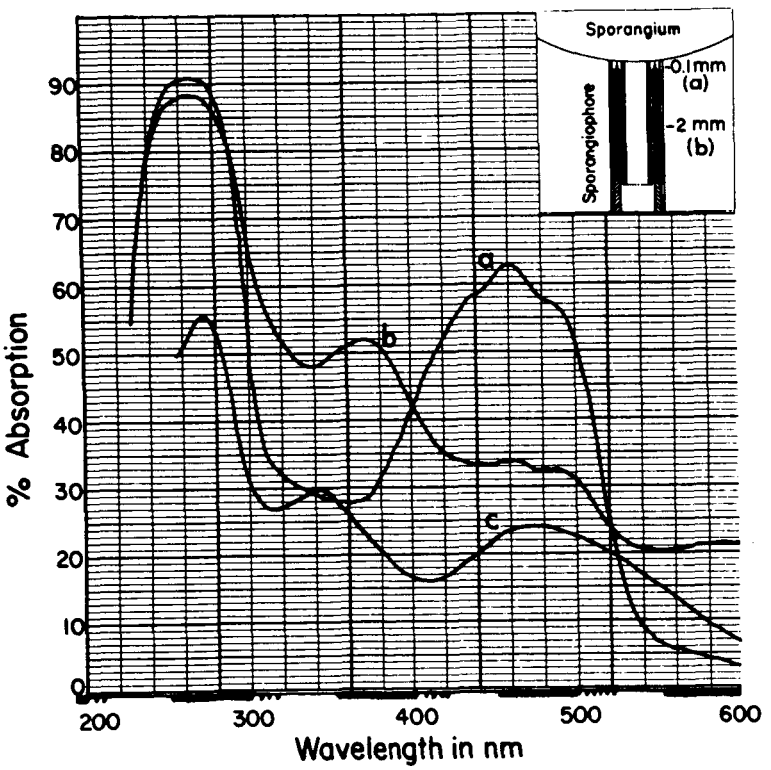



FIGURE 6. (a) absorption spectrum of sporangiophore, from 0.1 mm to 2 mm; stage IVb;
(b) absorption spectrum of sporangiophore, from 2 mm and down the sporangiophore; stage IVb;
(c) absorption spectrum of a crystal in sporangiophore stage I.

Phycomyces albino car-10 mutant, which responds as well as the wild-type to light stimuli, is deficient in β-carotene, and its spectra should be more informative. The absorption spectrum within the growing-zone of the albino car-5 and car-10 (Figure 7a) show spectra with absorption peaks near 230, 267 and 370 nm, which is a typical spectrum obtained in scanning throughout the growing-zone of the sporangiophore. No absorption peaks typical of β-carotene were found. It is important to reconcile these spectral findings to see more precisely the meaning of our absorption spectra.

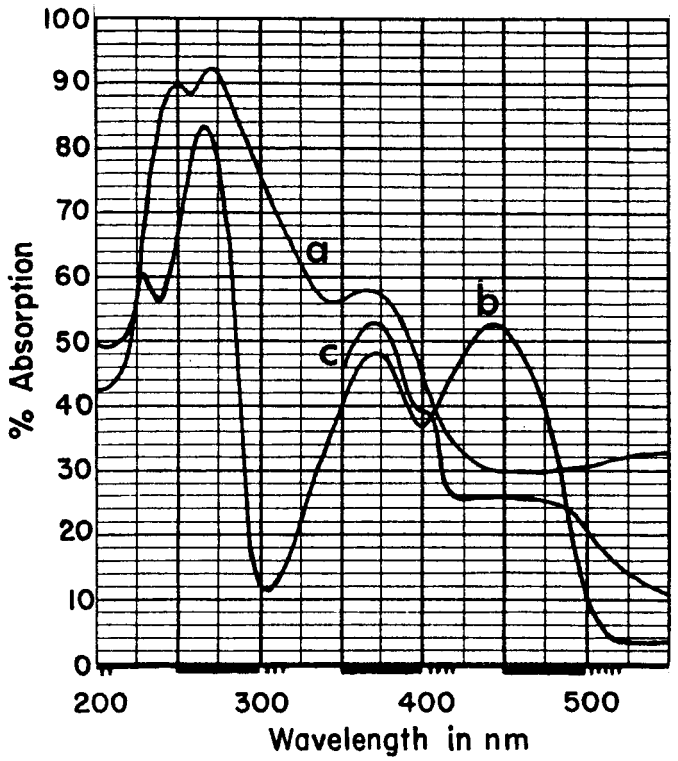



FIGURE 7. Absorption spectra.
(a) Phycomyces blakesleeanus car-10 absorption spectrum of sporangiophore;
(b) absorption spectrum of riboflavine;
(c) absorption spectrum of reduced flavine semiquinone.

Even though no detectable β-carotene spectra were observed by microspectrophotometry in the growing-zone of the albino car-10 and car-5, a carotenoid cannot be ruled out as the photoreceptor pigment. If all the β-carotene found in the sporangiophores were concentrated in the growing-zone, there would be $1.2 \times 10^{10}$ molecules. This is more than sufficient when compared to chlorophyll in chloroplasts and to rhodopsin in the retinal photoreceptors which contain from $10^6$ to $10^9$ pigment molecules.

If such concentrations were in the growing-zone it would have been detected by our microspectrophotometer, but no such concentrations of β-carotene were observed in the growing-zone of the albino mutants. Therefore, whatever β-carotene is present is most likely spread throughout the sporangiophore. What role β-carotene performs in this photoreceptor process is not clear, for it can act as a filter to screen or shade in the wavelength range of 400-500 nm, prevent photooxidation (destruction) at high light intensities, or it can participate as an accessory pigment molecule in the energy transfer process.

Neither the action spectra nor the absorption spectra of the growing-zone of Phycomyces have been sufficiently informative to clearly identify the primary photoreceptor molecule. Both are ambiguous enough to allow supporting interpretation for either a carotene or a flavine photoreceptor molecule.

Flavines. Recent evidence indicated that the flavines are probably involved in the photoreceptor process, and microspectrophotometry of stage IVb sporangiophores showed what appeared to be a reduced flavine spectrum. From these spectra (Figures 7b and c) it was very probable that Phycomyces sporangiophores should contain flavines. Therefore, it seemed that the role of flavines had been neglected, for no quantitative work was done to establish either the amount or the identity of the flavines present in the sporangiophore. The total flavines were determined using a fluorescence method. The results indicated that the sporangiophores of the stage IVb of the wild-type and car-10 contained an average of $13 \times 10^{12}$ and $5 \times 10^{12}$ flavine molecules respectively. Such concentrations are more than sufficient, as already indicated, when compared to the photosynthetic and visual photoreceptors.

Establishing the identity of these flavines is more difficult. The main flavines which were identified for the wild-type and the car-10 appear to be riboflavin, lumiflavin, lumichrome, flavine adenine dinucleotide (FAD) and flavine mononucleotide (FMN). How reasonable are these assertions about the sporangiophore? Indeed, it would seem unlikely to find the riboflavin molecule intact and in its pure state; yet the indications of lumichrome (or flavine semiquinone) greatly enhances its plausibility.

Electron Microscopy. Let us turn now to the microscopy of the sporangiophore, to see whether the spectra are associated with a particular cytoplasmic organelle.

For electron microscopy, the sporangiophores, stage IVb, were fixed in 1% $OsO_4$, pH 4.6, and sections from the light-growth zone were examined with the electron microscope. The electron micrographs showed numerous mitochondria, electron dense granules in various aggregated states and lipid globules (see cross-section of sporangiophore, Figure 8). None of these structures at present can be identified as the photoreceptor. However, crystalline microbodies from 2 μ to 10 μ were observed (Figures 8, 9, 10, 11 and 12). These crystals were chiefly located in the growth-zone 2-5 mm below the sporangium, where the photoreceptor is believed to lie. The distribution and frequency of occurrance of crystals in Phycomyces sporangiophores varied with the growth stage, strain, and the physical conditions for growth (see Figure 15).

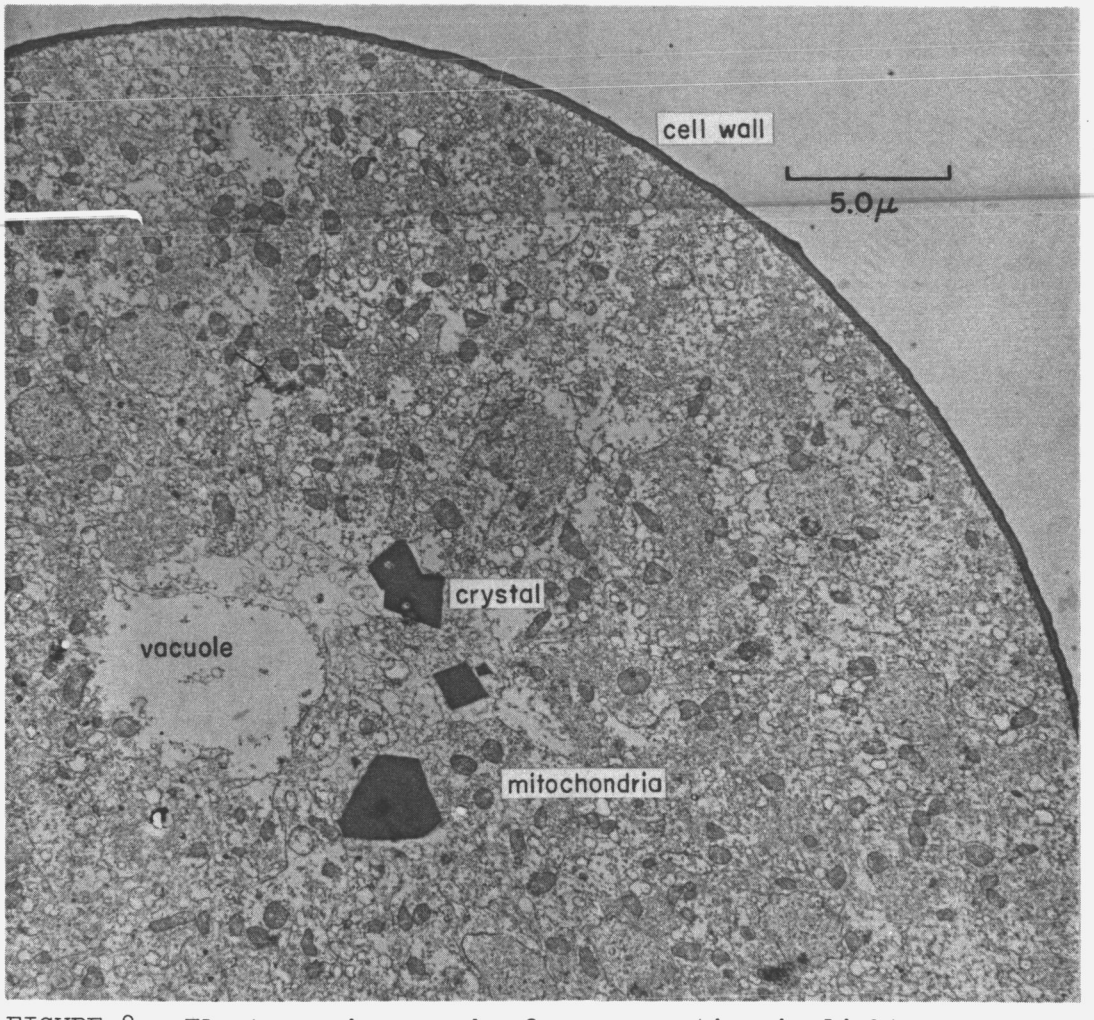



FIGURE 8. Electron micrograph of cross-section in light growth zone of Phycomyces blakesleeanus wild-type, stage IVb.

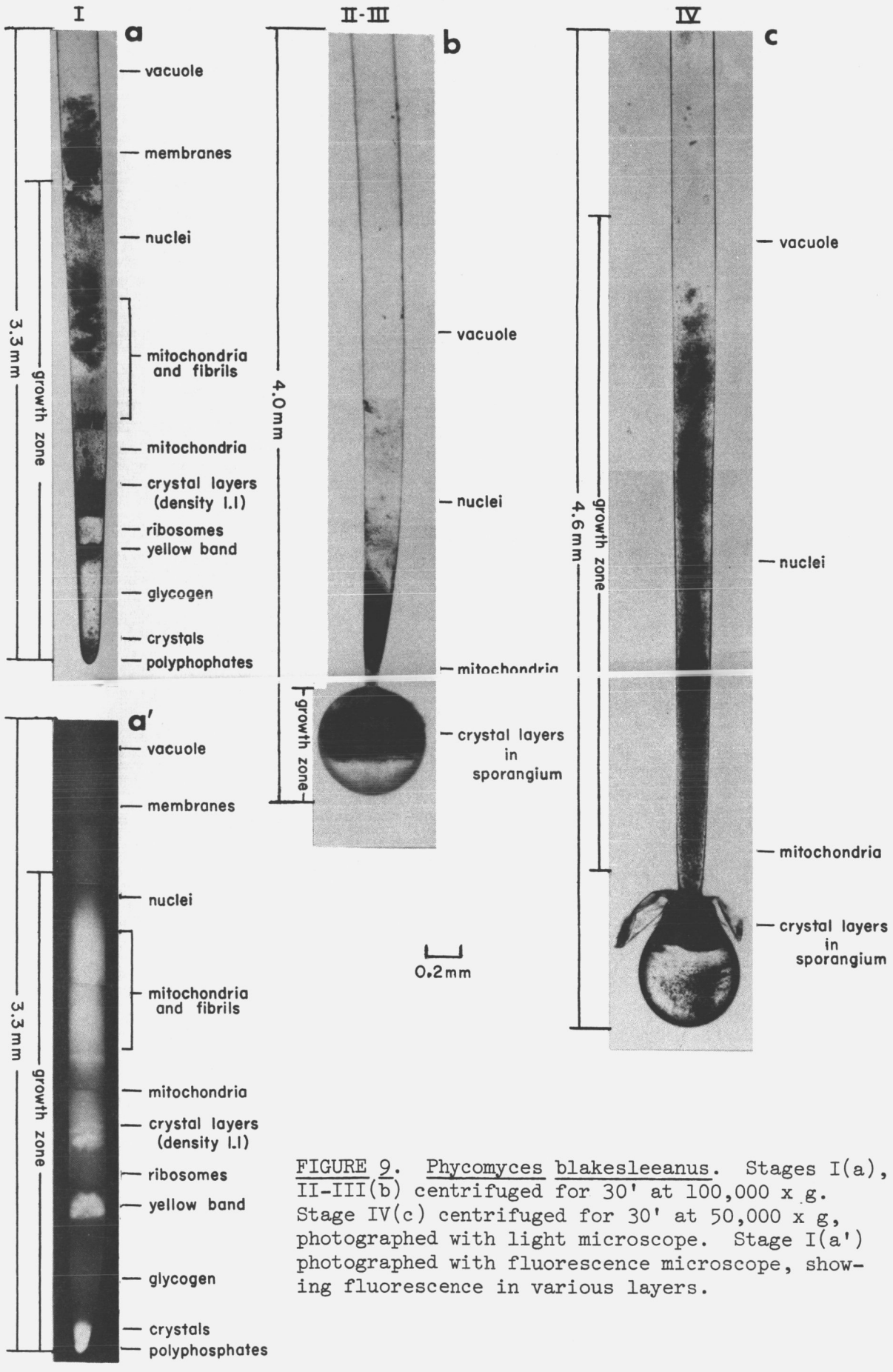



FIGURE 9. Phycomyces blakesleeanus. Stages I(a), II-III(b) centrifuged for 30' at 100,000 x g. Stage IV(c) centrifuged for 30' at 50,000 x g, photographed with light microscope. Stage I(a') photographed with fluorescence microscope, showing fluorescence in various layers.

Crystals. The identification of the crystals in the phototropic light-growth zone of stage IVb sporangiophores, and the possibility that they are the photoreceptors, made it necessary to isolate and chemically identify them.

It was found possible to centrifuge at high speed (to 100,00 x g for 30' to 1 hr) the whole sporangiophore. By this method, the cellular material became stratified according to their densities (Figure 9). At densities near 1.1 and 1.7, crystalline material could be observed. By puncturing the sporangiophore membrane, these crystal layers could be isolated. The isolated crystal fraction can be further clarified by centrifugation in 40% sucrose; in this way, free crystals are obtainable (see report by Dr. T. Ootaki for isolating crystals in a relatively pure state, pages 17-20).

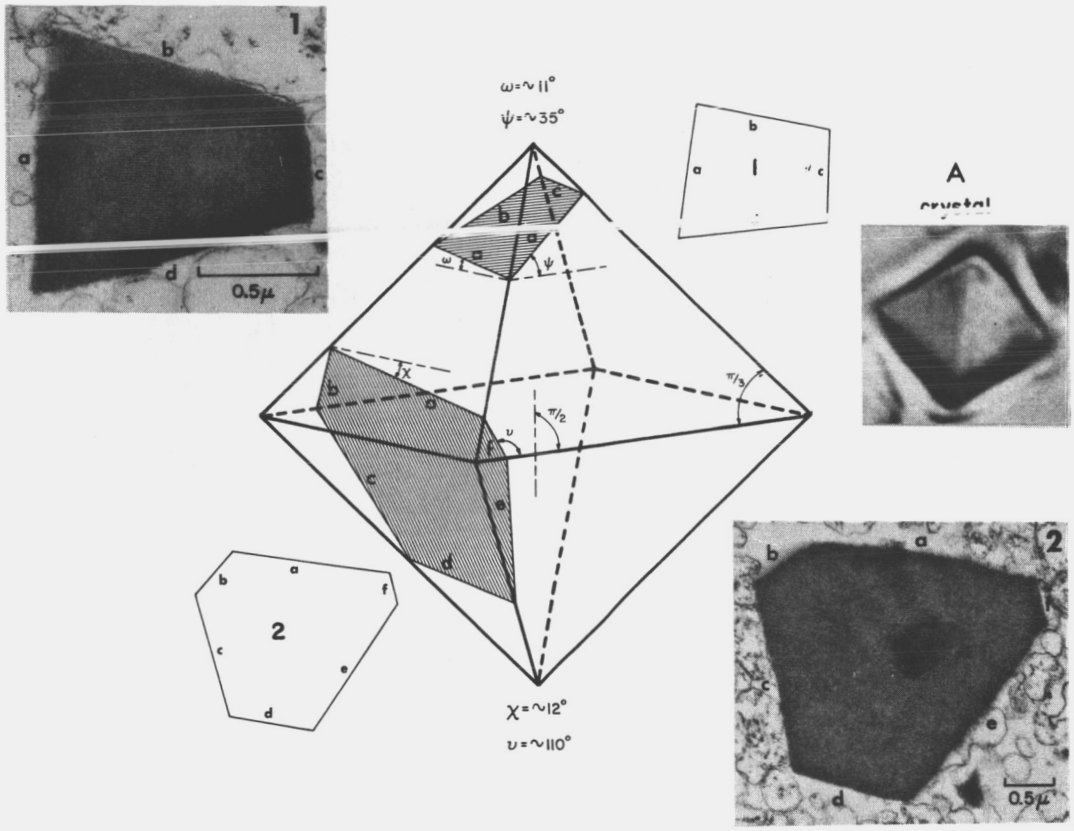



FIGURE 10. Isolated crystal, A, and octohedron model. 1 and 2, electron micrographs of sections through two different crystals. Angle of section cut, important for interpretation of optical diffraction patterns (Figures 11 and 12) and for establishing "unit cell," in molecular model (Figure 13).

These isolated crystals (from stage IVb) were then subjected to chemical analysis. Preliminary analysis of these crystals showed them to be about 80-88% protein and 10-12% lipid (as fatty acids). The high protein content of the crystals is attributable to its enzymic nature, for it had appreciable acid phosphatase and catalase activity.

The absorption spectrum of single crystals (stages I and IVb) obtained by microspectrophotometry showed absorption in the ultraviolet in the neighborhood of 280 nm and near 350 nm, and stage I had, in addition, a visible peak near 460 nm (Figure 6c). These are, in fact, the same absorption peaks found in the action spectrum and by microspectrophotometry of the stage IVb growing-zone (Figure 6a and b) and that of a flavine (Figure 7b and c).

Because of their small size, 2 to 10 μ, x-ray diffraction was not possible, and the method of optical diffraction of the electron micrographs of the sections through the fixed crystals as they occur in the sporangiophore were then obtained (see Figures 10, 11 and 12).

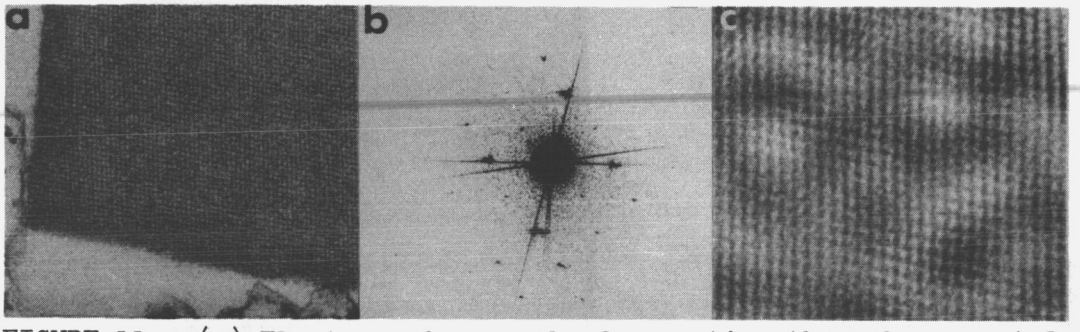



FIGURE 11. (a) Electron micrograph of a section through a crystal, "A" view. 38,150x. (b) Optical diffraction pattern of the crystalline area of (a). (c) Filtered image of (a), 195,000x.

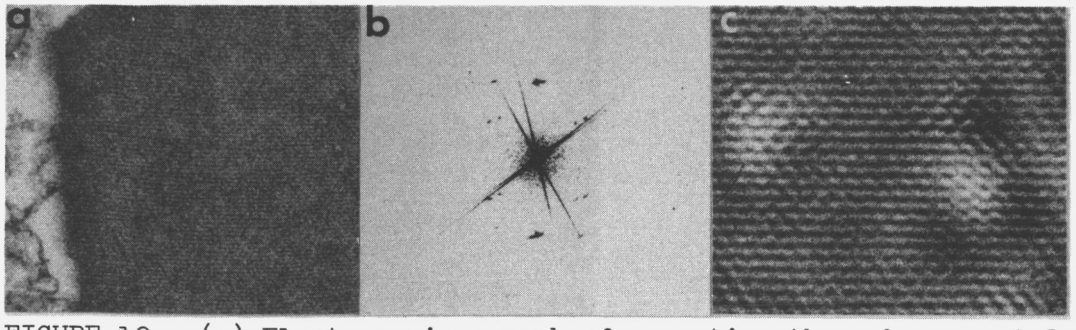



FIGURE 12. (a) Electron micrograph of a section through a crystal, "B" view. 34,240x. (b) Optical diffraction pattern of the crystalline area of (a). (c) Filtered image of (a), 195,000x.

The results of the optical diffraction of the crystal sections are illustrated in Figures 11a and b, and in Figure 12a and b. The filtered images are shown in Figures 11c and 12c. These data including that illustrated in Figure 10, led us to the schematized model in Figure 13. The crystal structure then consists of chains of discs 120 Å in height, which are in contact along the c-axis. A two-fold screw is parallel to the b-axis, and operates on an asymmetric unit consisting of two discs. The unit cell is monoclinic and belongs in the space group $P2_1$, with B=80°, a=240 Å, b=240 Å and c=260 Å. From the volume of any one disc, which is approximately $6.8 \times 10^5$ Å$^3$, the molecular weight was estimated to be of the order of 450,000. In each unit cell there are four discs.

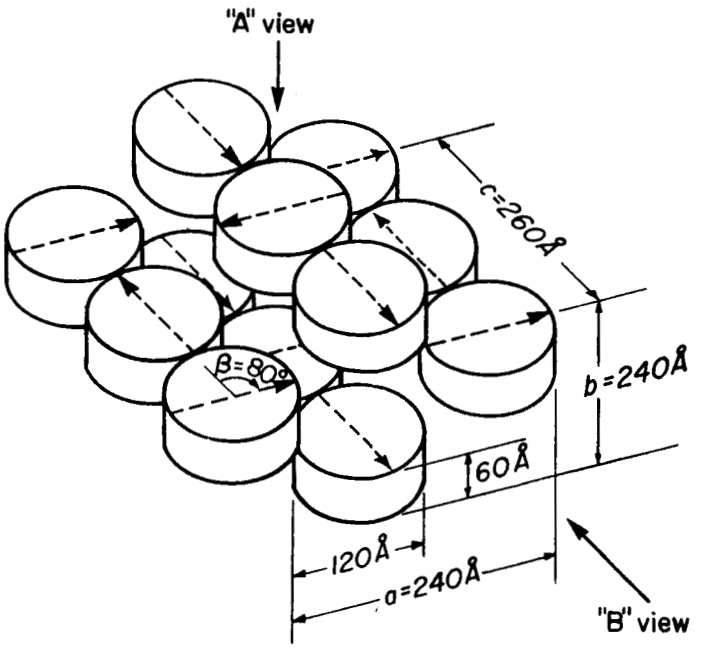



FIGURE 13. Model of unit cell and symmetry of the crystal of Phycomyces blakesleeanus.

The Photoreceptor. Even though chemical analysis of the isolated crystals from stage I and stage IV show considerable complexity, it is worth considering that these crystals could be the photoreceptor structure. A crystalline structure provides a large surface area for the photopigment, it brings molecules close together for interaction, and therefore, for energy transfer. If, indeed, our spectra of the light-sensitive sporangiophore and the crystals have some meaning, we can say that probably the photoreceptor molecule is a flavine which becomes reduced upon irradiation. Compare, for example, Figures 6b and c with Figure 7.

I would now like to raise the question -- do we have a comparable mechanism at the photochemical level between the phototropic response of the Phycomyces sporangiophore to that of the visual pigment system?

Although no one has found a photoreceptor pigment in the Phycomyces sporangiophore comparable to a rhodopsin, Meissner and Delbrück (Plant Physiol. 43:1279, 1968) did detect retinal in their extracted carotenoid fractions from the sporangiophore, but attributed this to the oxidation of β-carotene, the precursor for Vitamin A and hence retinal.

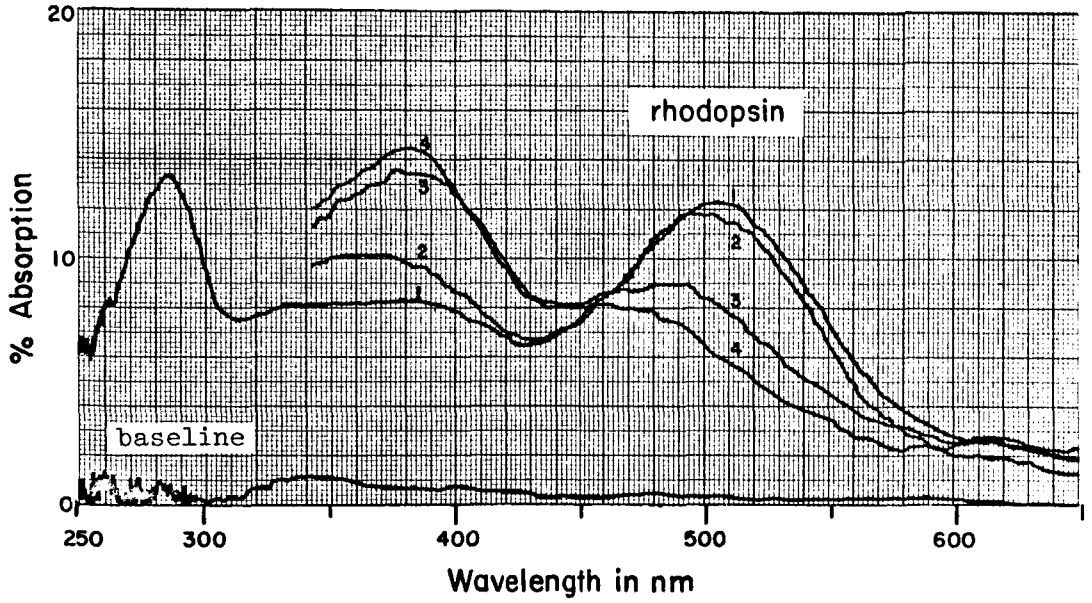



FIGURE 14. Absorption spectrum of an isolated frog retinal rod curve 1; spectra 2-4, after bleaching with green light (500 nm) for 15 sec. periods. Note the shift in spectra from rhodopsin, curve 1, to that of retinal, curve 4. The uv peak for opsin near 280 nm does not shift.

Therefore, I would like to turn to the visual process and the photochemistry of the retinal rod pigment rhodopsin of the eye. Spectroscopy of the freshly isolated retinal rod shows a typical rhodopsin absorption spectrum, Figure 14, curve 1. Upon irradiation with light, it bleaches (curves 2, 3 and 4). This bleaching is thought of as a conformational change in which the 11-cis retinal (Figure 2) is released from the rhodopsin complex to the all-trans retinal upon irradiation.

We can then ask, is there a similar phenomena observed in the sporangiophore and in the isolated crystal comparable to the spectroscopy of rhodopsin in the retinal rod upon irradiation?

By comparing the spectra of the photobleaching of a flavine to a reduced flavine or semiquinone (Figure 7) with that of the photobleaching of a rhodopsin (Figure 14), we see that the spectra of the flavines appear to have some similarities. That is, there is loss in absorbance or bleaching of the major peak in the visible region of the spectrum around 460 nm with an increase in absorbance of the 370 nm peak (Figure 7). Similarly, when rhodopsin is photobleached there is a loss in absorbance in the visible region 490-500 nm and an increase in absorbance around 370 nm (Figure 14).

As intriguing as this hypothesis is for the photoreceptor system in Phycomyces, which would be comparable to more highly developed visual sensory systems of animals, additional studies are necessary.

B. Isolation of Crystals from Phycomyces Sporangiophores. T. Ootaki.

In the Phycomyces sporangiophores, crystals were found in the wild-type G-5(+) and in the β-carotene deficient mutants, car-10 and car-5. Crystals were not identified in the vegitative hyphae, the storage vesicles, and the spores. Direct counting of crystals indicated that the wild-type sporangiophores contained more crystals than car-10 (which is fully phototropic) and much more than car-5 (which is not phototropic in dim light). I observed that the crystals were found in the cytoplasm near the tip and mainly in the vacuole. No specific accumulation of crystals at the apical region was revealed. In wild-type sporangiophores, the total number of crystals per sporangiophore consistently increased with the progress of the developmental stage and with increment of sporangiophore length until early stage IV, and then gradually decreased (see Figure 15). This was true for any sporangiophores cultured under different light conditions. In cultures grown in the

dark, the crystals started to decrease in numbers earlier than stage IV. The number of crystals per fresh weight of sporangiophores, however, was the highest in stage I and the lowest in stage IV. Therefore, the best yield of crystals is obtainable from stage I.

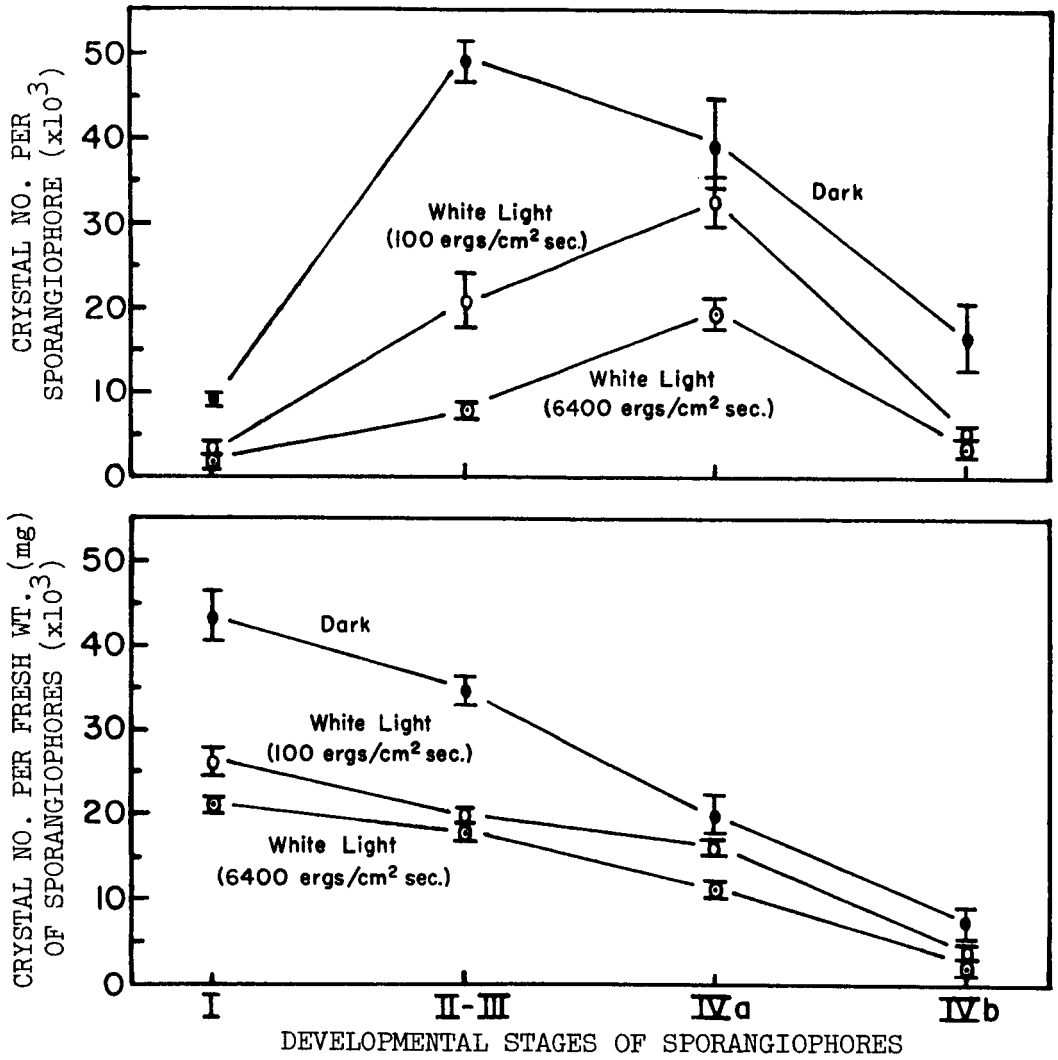



FIGURE 15. Number of crystals counted per sporangiophore found for Phycomyces grown in the light and in the dark for various growth-stages.

The number of crystals in the sporangiophores were found to be greatly dependent on the light intensities. For example, illumination with white light reduced the crystal content in sporangiophores at all stages, resulting in practically two-fold difference between dark- and light- (6400 ergs/$cm^2$-sec) cultures (Figure 15). Although no microscopical difference was detectable on the shape of crystals through all stages of sporangiophores, the crystal size seemed to be smaller in stage IV than stages I, II or III. In stage I, the crystals averaged about 8.5 μ in length, although a large variety of sizes was observed.

From these observations, the Phycomyces crystals appear to be related to light and are metabolically functional organelles. Therefore, they are not accumulated debris as a by-product of their metabolism. The metabolic function of these crystals in Phycomyces is still unknown.

Crystal Isolation and Properties. A method for isolating the crystals from the Phycomyces sporangiophores was worked out. The method is summarized in Table I. From 4 grams (wet weight) of sporangiophores, stage I, harvested from Phycomyces, wild-type (cultured in continuous white light, 100 ergs/ $cm^2$-sec) 8 mg of crystals were obtained. The specific gravity (density) of the crystals was found to be between 1.379 and 1.411. In the sedimentation, the main crystal fraction was always found to occur between 75 and 80% sucrose solution.

TABLE I. Isolation of crystals from Phycomyces sporangiophores.

Sporangiophores (washed with distilled water)
↓
Cut with scissors (in 0.1 M phosphate buffer, pH 6.0)
↓
Filtered through cheese cloth
↓
Filtrate
↓
Centrifuged (500 x g for 10 min.)
↓
Pellet | Supernatant (discarded)
↓
Layered over buffered 40% sucrose after suspended in buffer
↓
Centrifuged (500,000 x g for 45 min.)
↓
Pellet (crude crystal fraction) | Supernatant (discarded)
↓
Layered over double layers of buffered 75 and 80% sucrose after suspended in buffer
↓
Centrifuged (200,000 x g for 60 min.)
↓
Sediments at bottom of 80% sucrose (discarded) | Middle band between 75 and 80% sucrose | Buffer (discarded)
↓
Washed with buffer
↓
Crystals

In addition to the microscopical observations, the crystals showed no birefringence under a polarizing microscope, suggesting that the Phycomyces crystal was a regular octahedron and had properties of three equal crystallographic axes which crossed at right angles to each other, 24 symmetry elements, single refraction, and no interference figure (see Figure 10).

The question of whether these Phycomyces crystals consist of calcium oxalate or gallic acid was then considered, for these compounds are common components of fungal cells. It was found from chemical analysis that these crystals do not consist of calcium oxalate or gallic acid.

The spectrum of the isolated crystals showed a major absorption peak around 280 nm, indicating a protein. In addition, the crystals, after fixation with Carnoy's solution, stained positive for the mercury-bromphenol blue test, and the red-violet color for the biuret reaction. The protein concentration of these crystals was quantitatively estimated by a spectrophotometric method developed by Lowry (1951). The crystals were found to contain from 65-70% protein. The observation that these crystals developed a yellow color with conc. $HNO_3$ after boiling and then turned orange color with $NH_3$ after cooling (xanthoproteic reaction) suggested the possibility that the crystal protein contains tryptophane and aromatic amino acids (i.e., tyrosine and phenylalanine).

Although neither in situ crystals in sporangiophore cells nor isolated crystals showed detectable fluorescence under a fluorescence microscope, the mass of crystals in the crystal layers which were stratified in intact sporangiophore cell by ultracentrifugation at 100,000 g for 30 min showed a fluorescence (Figure 9a'), suggesting the possibility of the association of fluorescent substance(s) with the crystals.

C. Fluorescence of Phycomyces Sporangiophore Components.
Alfred J. Schultz, Jr.

The photoreceptor of Phycomyces must almost certainly show spectroscopic changes induced by light which are reversible in darkness. In view of this, the experiment reported by Berns, D.S. and J.R. Vaughn (Biochem. Biophys. Res. Comm. 39:1094, 1970) was partially repeated. However, our results differed from theirs. They reported reversible light-induced changes in the absorption and fluorescence excitation spectra in the phototropically effective regions of Phycomyces' action spectrum. In our experiments,

homogenates of Phycomyces blakesleeanus (wild-type) sporangiophores were fractionated by differential centrifugation. These fractions were examined by ordinary absorption spectroscopy in a Cary-14 spectrophotometer (0.0-2.0 O.D. slidewire). Subsequently, illuminated vs unilluminated difference spectra were taken of each of the fractions in the same spectrometer. Illumination of each sample was provided by white fluorescence tubes ($\lesssim$ 0.5 mwatts/cm$^3$) for 2 min immediately prior to measurement of the difference spectrum. In no case was a difference observed (detection limit $\lesssim$ 0.02 O.D.). However, absorption of these samples was low ($\sim$ 0.05 O.D. at 400-500 nm) and scattering high ($\sim$ 1.0 O.D. at 450 nm). A crystal fraction and a cell wall fraction were also examined with similar negative results. The crystal fraction has easily discernable shoulders at 232 nm and 265 nm, but no other observable absorption bands.

Next a supernatant fraction (78,000 x g; 30 min) was chosen on the basis of possessing the highest absorption to scattering in the region of interest (300-400 nm) for examination by fluorescence spectroscopy. Excitation spectra were recorded in an Aminco-Bowman spectrofluorometer. Emission was monitored at 580 nm. One very broad excitation peak was observed at 438 nm. The band shape was compatible with it being the sum of two bands. Illumination with a white fluorescent lamp (for about 5 min) bleached the excitation band, particularly the shorter wavelength components. But, unlike Berns and Vaughn's results, the bleaching was irreversible. In fact, the bleaching continued in darkness. The final excitation band maximum was found at 450 nm (after 5 minutes in light and 30 minutes in darkness). The absorption spectrum of this sample had a strong, broad shoulder from about 650-680 nm and an ultraviolet peak at 257 nm.

Pure riboflavin dissolved in distilled $H_2O$ was observed by us to have absorption maxima at 445, 374, 267 and 222 nm. The excitation maxima of the riboflavin solution were at 270, 373, 450 and 462 nm. Emission was monitored at 520 nm, the position of the riboflavin solution fluorescence peak we observed.

Phytofluene, a precursor in the synthesis of carotenoids, is also found in Phycomyces (Goodwin, T.W.; Biochem. J. 50:550, 1952). It possesses a strong greyish-green fluorescence. In petroleum ether its absorption maxima are at 330, 348 and 367 nm. The 340 nm excitation component observed by Berns and Vaughn might originate from phytofluene.

We observed stage I sporangiophores under the fluorescence microscope. Our purpose was to determine which portions of the sporangiophore are fluorescent. An overall green fluorescence was observed. It was especially intense from either the tonoplast membrane or the vacuolar sap. After centrifugation of a whole sporangiophore the various layers were also fluorescent (Figure 9a'). The mitochondrial layer had an especially intense green fluorescence. The crystal layer had a moderate amount of green fluorescence; however, no fluorescence could be observed originating from discernable crystals. This was true both inside the sporangiophore where clumps of crystals could be seen and outside after the cell wall had been ruptured at the crystal layer. Crystals of stage IV sporangiophores were also examined inside the sporangiophore, in squeezed-out cell sap, and those that were isolated. Again, no fluorescence was observed originating from the crystals.

It is well known that the binding of a chromophore to its carrier molecule, e.g., flavin to protein, often greatly reduces the chromophore's fluorescence yield. With this in mind, we decided to dissolve crystals in 0.1 N NaOH. Stage I sporangiophores were centrifuged. After centrifugation, they were found to have the same distribution of fluorescence as described above for stage I sporangiophores. Then 0.1 N NaOH was added. The layers gradually disintegrated and their fluorescence increased as the base permeated the sporangiophore. Crystals could be observed to be dissolving near the edge of a layer (it was not clear whether the crystal and mitochondrial layer had merged or whether the crystal layer had already largely dissolved). No fluorescence could be observed coming from the crystals or the immediate area surrounding them.

D. The Effect of Light upon the Fruiting Response of Phycomyces.
A.J. Schultz, Jr. and T. Ootaki.

The influence of white light intensity upon the formation of the fruiting stalk (sporangiophore) and the fruiting body (sporangium) of Phycomyces was investigated. Flowering, the analogous response of higher plants, is known to depend upon the light conditions. The flowering response is mediated by the pigment phytochrome (Figure 16). The relationship between light and the fruiting response in a fungi as primitive as Phycomyces has never been investigated.

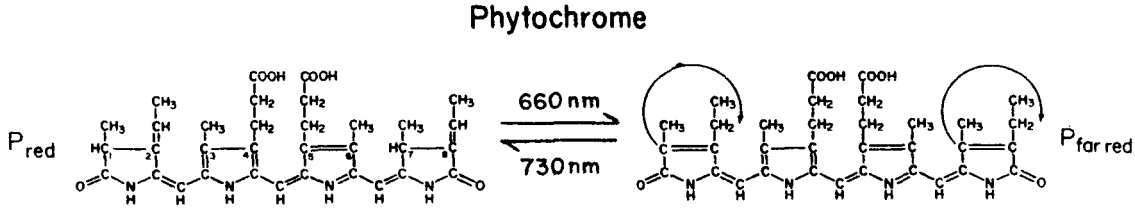



FIGURE 16. Phytochrome, $P_{red}$ and $P_{far\ red}$ forms.

Phycomyces blakesleeanus wild strain G-5(+) was cultured at 23°C ± 1°C on 1.5% Sabouraud Dextrose Agar. Illumination from the time of innoculation to the time of sporangium formation was provided by a white fluorescent lamp (Westinghouse Cool White, F40CW). Intensities at the culture position were adjusted by varying both the distance of the boxes containing the culture from the light source and the number of frosted plexiglas covers over the boxes.

The intensities used were about 50, 100, 200, 400, 800, 1600 and 3200 ergs/cm$^2$-sec. Controls were cultured in complete darkness. At the time of sporangium formation (stage II-III) the length of the sporangiophore was measured. In each experiment, usually about 100 sporangiophores were measured for each intensity. At least three separate experiments were done at each light intensity.

Sporangiophore length at the time of sporangium formation does show a definite dependence upon light intensity (Table II). However, the fruiting response's requirement for light is not absolute; i.e., it did occur in total darkness. But sporangiophore initiation did not take place as consistently in darkness as it did when the cultures were illuminated. In addition, when sporangiophores did form, the length of the sponragiophore at stage II-III was substantially longer in darkness than in the light. The mean (stages II-III sporangiophores) lengths ranged from 31.1 mm in darkness to 8.6 mm under an illumination intensity of 1600 ergs/cm$^2$-sec (Table II). The saturation intensity for the fruiting response appears to be of the order of 1000 ergs/cm$^2$-sec. The fruiting response to light intensity does not follow a simple first or second order relationship. The series of events leading from light detection to fruiting response is undoubtedly very complex.

The presence of light only promotes the fruiting response of Phycomyces. Thus, spontaneous dark excitation of the photoreceptor could exist. Light would greatly accelerate the excitation of the photoreceptor and hence ultimately the fruiting response would be accelerated.

TABLE II. The variation between stage II-III (time of sporangium formation) sporangiophore length with light intensity in Phycomyces blakesleeanus.

| Light intensity ergs/cm$^2$-sec | Stage II-III Sporangiophore Length (mm) |
|---|---|
| 0 | 31.1 $\pm$ 0.5 |
| 53 | 21.5 $\pm$ 1.2 |
| 116 | 15.5 $\pm$ 1.9 |
| 199 | 14.2 $\pm$ 2.1 |
| 407 | 12.0 $\pm$ 1.4 |
| 790 | 11.0 $\pm$ 0.8 |
| 1690 | 8.6 $\pm$ 1.2 |
| 3268 | 8.7 $\pm$ 1.1 |

## IV. Expeditions

### A. Darwin Station, Galapagos Islands

As a part of our photobiological research program on the origins and evolution of life, Professor Wolken repeated a part of Darwinia by participating in an expedition following by sea that of Darwin and the Beagle, to the Galapagos Islands, Ecuador. He was a guest of UNESCO at the Darwin Research Station, Academy Bay, Santa Cruz, Galapagos Islands, from May 15 to June 21, 1970.

The main purpose was to observe the diverse forms of life on the various islands in the Galapagos chain. Because of the Laboratory's interest in the eye, its development, optics and vision, attention was directed to the eyes of these animals.

Of particular interest for study was that of the "Four-eyed" Blenny (Dialommus fuscus). This fish is amphibious in behavior. These eyes have a partitioned cornea, which adjusts for land and sea vision. The behavior of these fish was observed and photographed, and their eyes fixed for further study. The anatomical study of these eyes are now being carried on in our Laboratory by microscopy and electron microscopy.

### B. Smithsinian Institutions, Tropical Research Station, Panama

From June 21 to July 1, 1970, further field studies were continued at the Tropical Research Station, Smithsonian Institution, Balboa Heights, and the Island of Barra Colorada, Panama. Much of the work here centers on life in a tropical forest. Of interest were the insects, spider monkey, ant eaters, sloths and armadillos. The sloth, because of its very slow response, is an unusually interesting animal for studies of behavior and the brain.

The various researches undertaken on these expeditions will be incorporated in a book on The Photoreceptor System and Evolution.

## V. The Laboratory

### A. Educational Programs

The Biophysical Research Laboratory is an integral part of the Biological Sciences program at Carnegie-Mellon University. In addition to carrying on a program of research in photobiology, the Laboratory serves in an educational capacity, as well. Research facilities and guidance are provided for a number of graduate students and for undergraduate senior research projects. The Laboratory also serves for interdisciplinary training with the Biotechnology program. Professor Wolken has served on the Engineering, Biotechnology, and Physics Ph.D. Thesis Committees for Dr. T. Vogel, Dr. T.A. Krouskop, Dr. D. Bellavia, and Mr. Kevos Spartalian.

Seminars and tutorials for graduate and undergraduate students are organized in close association with the research of the Biophysical Research Laboratory. In addition, Professor Wolken teaches a course in Biophysics (S/GS 557-558), and has taught a course, Science Systems (A-596), for non-science majors. During the 1970-1971 academic year, Dr. Wolken taught undergraduate (S/257-258) and graduate (GS/757-758) level Biophysics I and II.

In the teaching and research projects, our Visiting Research Fellows participate as tutors. The Research Fellows who have served in this capacity are Dr. G. Marak, Dr. T. Ootaki and Dr. A.J. Schultz, Jr. In addition, the Laboratory staff has instructed students in the use of biophysical instruments and supervised undergraduate student research projects. Mr. G.J. Gallik, Mr. R.G. Florida and Mr. O.J. Bashor, Jr. have participated in the National Science Foundation pre-college summer instructional program, as well.

It is hoped that these various educational participations foster an interest in science and research. Above all, it is felt that the environment of the Laboratory encourages an enthusiasm toward creative thinking at a crucial stage in the development of scientific careers.

Opportunities and encouragement are given to the research staff of the Laboratory to further their own interests, to continue their studies towards advanced degrees, and to work at other research laboratories or universities for periods of time to broaden their own scope.

Since the establishment of the Laboratory in 1953, the granting of post doctoral research fellowships has been a continuing policy. Visiting Research Fellowships have been provided for research scholars and medical students in 1970-1971 through the generosity of the Pennsylvania Lions Sight Conservation and Eye Research Foundation, Inc., and the Scaife Family Charitable Trusts of Pittsburgh. These Fellows help provide the stimulating environment so necessary for productive research.

## B. Research Facilities

The Biophysical Research Laboratory is located in the Margaret Morrison Carnegie College Building. It occupies 5,000 square feet of well-equipped laboratories for biology, biochemistry and biophysics -- including facilities for chemistry, electrophysiology, electron microscopy, magentics, optics, and growth chambers for cell and tissue culture studies. In addition, there is a greenhouse for the cultivation of plants and the preservation of botanical material.

These research laboratories include equipment for performing preparative and analytical ultracentrifugation, electron microscopy, spectroscopy, optical diffraction, electrophoresis, instrumentation for radioactive tracer analyses, magnets of various strengths and electronic devices. To service the laboratories there is an experimental instrument shop, photographic darkrooms, specialized library and offices.

The Laboratory staff has available the use of the collection of the Hunt Botanical Library, Carnegie-Mellon University, and the natural history collections of the Carnegie Museum. Through cooperative arrangements with the Phipps Conservatory of Pittsburgh, special botanical material can be made available. For studies in visual physiology, cooperative arrangements can be made with the clinical and research laboratories of the Eye and Ear Hospital, Department of Ophthalmology, University of Pittsburgh School of Medicine.

## VI. Publications

### A. Research

Gruen, H.E. and T. Ootaki. 1970. Regeneration on Phycomyces Sporangiophores. I. Induction by decapitation and basal cutting on sporangiophores attached to mycelium and after isolation. Can. J. Bot. 48:55-65.

Ootaki, T. and H.E. Gruen. 1970. Regeneration on Phycomyces Sporangiophores. II. Regeneration on isolated segments from different positions along the sporangiophore axis. Can. J. Bot. 48:95-109.

Schultz, A.J., Jr. 1970. The development and organization of photosynthetic pigment systems; Ph.D. Thesis; Lawrence Radiation Laboratory, University of California (Berkeley); UCRL-20202; November, 1970.

Wolken, J.J. 1970. "Cell and Photoreceptor Membranes," In Proceedings of the Conference on the Physical Principles of Biological Membranes. Snell, F., Wolken, J.J., Iverson, G.J. and Lam, J., eds. Gordon and Breach Science Publishers, New York. pp. 365-382.

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Wolken, J.J. 1971 (in press). "Photobiology," In Enciclopedia Medica Italiana. Vella, L., ed. Rome, Italy.

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Wolken, J.J. 1971 (in press). "Microspectrophotometry," In Microscopy and Microtechnique, Gray, P., ed. Van Nostrand Reinhold Company, New York.

Wolken, J.J. 1971 (in press). "Phycomyces: A Model Photosensory Cell," In Proceedings of the Conference on the Physical Principles of Neuronal and Organismic Behavior. Conrad, M. and Mager, M., eds. Gordon and Breach Science Publichers, New York.

Wolken, J.J. 1971 (in press). "Photodynamics: The Chloroplast," In Phytochemistry. Miller, L.P., ed. Van Nostrand Reinhold Company, New York.

Morgan, R.S., Czeto, A.R. and Wolken, J.J. 1971 (in press). The unit cell and symmetry of the Phycomyces crystals.

### B. Books

Wolken, J.J. Euglena: an experimental organism for biochemical and biophysical studies. Rutgers University Press, New Brunswick, N.J. 1961

Wolken, J.J. Vision: biochemistry and biophysics of the retina. Charles C. Thomas Publishers, Springfield, Ill. 1966.

Wolken, J.J. Euglena: an experimental organism for biochemical and biophysical studies. Second Revised Edition. Appleton-Century-Crofts, New York, N.Y. 1967.

Wolken, J.J. Photobiology. Reinhold Publishing Company, New York, N.Y. 1967.

Wolken, J.J. Invertebrate Photoreceptors: a comparative analysis. Acadenic Press, New York, N.Y. 1971.

Edited Works

Photoreception. 74:161-406. Wolken, J.J., ed., New York Academy of Sciences. New York, N.Y. 1958.

Physical Principles of Biological Membranes. Snell, F., J.J. Wolken, G.J. Iverson and J. Lam, eds. Gordon and Breach Science Publishers, New York, N.Y. 1970.

C. Papers Presented at Seminars and Meetings

February 25-27, 1970. Fourteenth Annual Meeting, Biophysical Society. Baltimore, Md. "Isolation of Crystals from Phycomyces." Gallik, G.J., P.J. Dubash and J.J. Wolken.

April 14-17, 1970. Fifty-Fourth Annual Meeting of the Federation of American Societies for Experimental Biology. Atlantic City, N.J.

October 15, 1970. Department of Biological Sciences Seminar, Carnegie-Mellon University. Pittsburgh, Pa. "Arthropod Vision." Wolken, J.J.

October 17, 1970. Research Council of the Pennsylvania Lions Sight Conservation and Eye Research Foundation, Inc. Pittsburgh, Pa. "Optical Devices for the Visually Handicapped." Wolken, J.J.

November 4-6, 1970. Pittsburgh Diffraction Conference: Session on Electron Microscopy. Pittsburgh, Pa. "Phycomyces Crystals." Wolken, J.J.

November 10-12, 1970. Centennial Conferences on Visual Science. Ohio State University. Columbus, Ohio.

November 19-21, 1970. Tenth Annual Meeting of American Society for Cell Biology. San Diego, Cal. "Phototropism and the Nature of Crystals in Phycomyces." Wolken, J.J.

December 16-18, 1970. Conference on Physical Principles of Neuronal and Organismic Behavior. Center for Theoretical Studies, University of Miami. Coral Gables, Fla. "Phycomyces: A Model Photosensory Cell." Wolken, J.J.

December 22, 1970. Mote Marine Research Laboratory. Sarasota, Fla. Discussion, "Retinal Photoreceptors of the Dogfish Eye." Wolken, J.J.

January 11, 1971. Smithsonian Institution, Radiation Biology Laboratory. Rockville, Md. Seminar: "Development of the Photoreceptor System of Plants and Animals." Wolken, J.J.

February 15-18, 1971. Biophysical Society Meeting, Fifteenth Annual. New Orleans, La. "Phototropism: The Structure and Molecules of the Photoreceptor in Phycomyces." Wolken, J.J., T. Ootaki and A.J. Wolken.

March 1, 1971. Department of Biophysics, Michigan State University. East Lansing, Mich. Seminar: "Unicellular Organisms as Models for Sensory Behavior." Wolken, J.J.

April 22, 1971. Center for Theoretical Biology, State University of New York at Buffalo. Amherst, N.Y. Seminar: "Unicellular Organisms as Models for Sensory Behavior." Wolken, J.J.

April 29 - May 1, 1971. Symposium on Excitable Membranes. University of Virginia School of Medicine. Charlottesville, Va.

May 10-14, 1971. Biophysics Department, Dartmouth College. Hanover, N.H. Series of lectures on "Visual Pigments and Photoexcitation." Wolken, J.J.

July 14-28, 1971. Phycomyces Genetics Workshop. Laboratory for Quantitative Biology, Cold Spring Harbor. Long Island, N.Y. Ootaki, T.

July 31 - August 6, 1971. Phycomyces Genetics Workshop. Laboratory for Quantitative Biology, Cold Spring Harbor. Long Island, N.Y. Wolken, J.J.

September 14-17, 1971. First European Biophysics Congress. Baden, Austria. "Phycomyces Crystals: Their Structure and Function in the Cell." Wolken, J.J.

The Annual Reports of the Biophysical Research Laboratory, Volumes I-XVI, 1953 to 1971, reprints of publications and other related published works, are available upon request to the Laboratory. Published books and edited works should be ordered directly from the publishers.

Acknowledgements

The Biophysical Research Laboratory is most appreciative of the support it has had over these many years from the National Council to Combat Blindness, Inc., New York, N.Y.; The National Institutes of Health Institute of Neurological Diseases and Blindness, Bethesda, Md.; The National Science Foundation, Washington, D.C.; The Rachel Mellon Walton Foundation of Pittsburgh, Pittsburgh, Pa.; and The Kresge Foundation, Detroit, Mich.

In the year 1970-1971, the Laboratory would like to thank the National Aeronautics and Space Administration (NASA), Washington, D.C.; The Pennsylvania Lions Sight Conservation and Eye Research Foundation, Inc., Harrisburg, Pa.; and The Scaife Family Charitable Trusts of Pittsburgh, Pittsburgh, Pa.

June 11, 1971
date

Jerome J. Wolken
Jerome J. Wolken